Printed in Great Britain
J. Reprod. Fert. (1985) **73**, 411–424

Appearance of β-hexosaminidase and other lysosomal-like enzymes in the uterine lumen of gilts, ewes and mares in response to progesterone and oestrogens

P. J. Hansen*†, F. W. Bazer† and R. M. Roberts*

**Department of Biochemistry and Molecular Biology and †Department of Animal Science, University of Florida, Gainesville, Florida 32610, U.S.A.*

Summary. In one experiment, ovariectomized gilts were treated with corn oil (vehicle), progesterone, oestradiol-17β or both steroids. While oestradiol treatment did not stimulate enzyme activity in uterine flushings relative to vehicle-treated animals, gilts treated with progesterone had elevated amounts of all enzymes measured. Progesterone was less effective when co-administered with oestradiol-17β. Enzymes were not equally stimulated by progesterone. For example, there was a 909-fold increase in acid phosphatase activity in uterine flushings and a 304-fold increase in β-*N*-acetylglucosaminidase, but only a 10-fold increase in β-glucosidase. Endometrial explants from gilts synthesized and secreted radiolabelled β-*N*-acetylglucosaminidase, suggesting that at least some lysosomal enzymes enter the uterus through secretory processes. In other experiments, changes in β-*N*-acetylglucosaminidase in uterine fluids of mares and ewes treated with hormonal regimens similar to those given to the gilts were evaluated. Treatment with the combination of progesterone and oestrogen stimulated accumulation of the enzyme relative to that in vehicle-treated animals. The biochemical properties of porcine β-*N*-acetylglucosaminidase were examined in detail. Properties of the uterine enzyme were similar to reported values for lysosomal hexosaminidase. These included molecular weight (82 000–89 000), pH optimum (pH 4·4), presence of two isomers (isoelectric points of 5·5 and 8·0) and ability to hydrolyse substrates for glucosaminidase and galactosaminidase. We conclude that steroids induce the accumulation of lysosomal enzymes in the uterine lumen. The degree of stimulation differed between enzymes, suggesting that those enzymes stimulated to the greatest extent may play an important role in pregnancy.

Introduction

It is well known that lysosomal hydrolases are present in endometrial tissues of the uterus (Murdoch & White, 1968; Linford & Iosson, 1975; Rosado, Mercado, Gallegos, de los Angeles Wens & Aznar, 1977; Sengupta, Roy & Manchanda, 1979; Findlay *et al.*, 1981; Rahi & Srivastava, 1983). Lysosomal enzymes may also be secreted by the uterine endometrium since enzymic activity characteristic of lysosomes has been found in uterine flushings of cows (Roberts & Parker, 1974), ewes (Roberts, G.P., Parker & Symonds, 1976a), pigs (Roberts, R. M., Bazer, Baldwin & Pollard, 1976) and humans (Roberts, G. P., Parker & Henderson, 1976b). In addition, uteroferrin, a purple-coloured protein secreted in copious amounts by the pig endometrium, has many properties characteristic of a class of lysosomal acid phosphatases (Schlosnagle, Bazer, Tsibris & Roberts, 1974; Schlosnagle, Sander, Bazer & Roberts, 1976), and carries mannose-6-phosphate, the so-called lysosomal recognition marker, on its carbohydrate chain (Baumbach, Saunders, Bazer & Roberts, 1984). The functions of secreted lysosomal enzymes are unclear, although they have been postulated to be involved in conceptus–endometrial attachment, sperm capacitation and removal

of the zona pellucida (Roberts & Parker, 1974; Rahi & Srivastava, 1983; Roy, Sengupta & Manchanda, 1983). The finding that uteroferrin, while being a lysosomal phosphatase, functions *in utero* as an iron-transport protein (Roberts & Bazer, 1980; Buhi, Ducsay, Bazer & Roberts, 1982), raises the possibility that other lysosomal-like proteins may have functions during pregnancy other than their enzymic ones.

The present experiments were conducted to test several hypotheses. We first measured levels of several lysosomal-like enzymes in uterine flushings of ovariectomized, steroid-treated gilts to determine whether accumulation of all lysosomal enzymes is stimulated equally by progesterone or whether differential amplication of enzymic activities occurs. In several other experiments, we examined more closely one enzyme, β-*N*-acetylglucosaminidase, which is present in large amounts in uterine flushings. We evaluated whether the enzyme found in uterine fluid is similar to lysosomal β-hexosaminidase, whether the endometrium releases the enzyme during culture and whether induction of the enzyme by progesterone also occurs in ewes and mares.

Materials and Methods

Materials. Steroids, lysozyme, sodium taurocholate, Protein A–Sepharose 4B, concanavalin A–Sepharose 4B and most enzyme substrates were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. Other enzyme substrates were obtained from Calbiochem (San Diego, CA, U.S.A.) and Pierce Chemical Co. (Rockford, IL, U.S.A.). Chromatography supplies were purchased from Pharmacia (Piscataway, NJ, U.S.A.) and Whatman (Clifton, NJ, U.S.A.). Amersham Corp. (Arlington Heights, IL, U.S.A.) supplied L-[4,5-^{3}H]leucine at a specific activity of 130 Ci/mmol. Goat anti-rabbit IgG was obtained from Miles-Yeda, Rehovot, Israel. Dr Gary Sahagian and Dr Elizabeth Neufeld (NIH, Bethesda, MD, U.S.A.), generously donated rabbit antiserum to human β-hexosaminidase and Dr Nathan Sharon (Weizmann Institute, Rehovot, Israel) kindly provided 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine (CNAG). Sources of reagents for endometrial explant culture and electrophoresis have been detailed elsewhere (Godkin, Bazer, Moffatt, Sessions & Roberts, 1982).

Animals and treatments. Experiments were done to evaluate steroidal induction of lysosomal-like enzymes in the uterine lumen. Twelve crossbred gilts (about 110 kg each) were ovariectomized on Day 4 of the oestrous cycle (oestrus = Day 0) and randomly assigned to receive daily injections of vehicle (corn oil), oestradiol-17β (100 μg/day), progesterone (200 mg/day) or both steroids, beginning on the day of ovariectomy. There were 3 gilts per group. After 30 days of treatment, uterine secretions were recovered as previously described (Bazer, Sharp & Roberts, 1978) by flushing the uterus with 0·9% (w/v) NaCl. In another experiment, 16 long-term (>6 months) ovariectomized pony mares (about 200 kg each) were injected daily with vehicle (sesame oil, N = 4), oestradiol benzoate (10 mg/day; N = 5), progesterone (150 mg/day; N = 3) or both steroids (N = 4) for 28 days, at which time the uterus was flushed as described by Zavy *et al.* (1982). Eight Western Whiteface ewes (about 50–60 kg each) were also ovariectomized, on Day 4 of the oestrous cycle, and a ligature placed around one uterine horn using the procedure of Bazer *et al.* (1979). After ovariectomy, ewes were injected daily with vehicle (corn oil; N = 2), oestrone (5 μg/day; N = 3) or oestrone (5 μg/day) plus progesterone (50 mg/day; N = 3). At 30 days after surgery, uterine fluid was aspirated from the ligated uterine horn after exposing the uterus by laparotomy.

Another experiment was designed to study protein accumulation in the uterus of gilts during the oestrous cycle and early pregnancy. Uteri from 34 crossbred gilts, pregnant or non-pregnant, were flushed with 0·9% (w/v) NaCl at Days 6, 10, 11, 12, 14 or 16 after oestrus.

The biochemical properties of β-*N*-acetylglucosaminidase were analysed by using uterine flushings of 'pseudopregnant' gilts as a source of the enzyme. Non-pregnant animals were made pseudopregnant by injecting them (i.m.) with 5 mg oestradiol valerate on Days 11 to 15 after

oestrus, as described by Frank, Bazer, Thatcher & Wilcox (1977). Uterine flushings were collected from these animals on Day 90 or 110 after oestrus.

Endometrial cultures. The procedures of Basha, Bazer & Roberts (1979, 1980) were used to culture endometrial explants of a pseudopregnant gilt in a modified minimal essential medium described by Masters *et al.* (1982). Briefly, 500 mg minced endometrium were cultured in 15 ml medium containing 100 μCi L-[4,5-^{3}H]leucine for 24 h at 37°C under an atmosphere of 50% O_2, 45% N_2 and 5% CO_2. Cultures were maintained in the dark on rocking platforms.

Culture medium was dialysed against 10 mM-Tris–HCl buffer (pH 8·2) and run through a carboxymethylcellulose (CM-cellulose) column (equilibrated in the same buffer) to remove uteroferrin and other basic proteins. The material not binding to the column was used for immunoprecipitation of β-*N*-acetylglucosaminidase or for CNAG-Sepharose chromatography.

Enzyme assays. Each enzyme was assayed at its pH optimum, experimentally determined for each of the enzymes found in uterine flushings. Acid phosphatase (EC 3.1.3.2) was assayed under reducing conditions as described by Schlosnagle *et al.* (1974, 1976) using *p*-nitrophenyl phosphate as a substrate. Arylsulphatase (EC 3.1.6.1) was assayed by incubating 100 μl of sample, 200 μl 0·2 M-acetate buffer (pH 5·5) containing 0·1% (w/v) bovine serum albumin, and 200 μl 5 mM-nitrocatechol sulphate at 37°C for 30 min. The reaction was stopped by the addition of 1 ml 0·5 M-carbonate buffer (pH 10·3) and free 4-nitrocatechol was determined by measuring absorbance at 515 nm. The cathepsin D (EC 3.4.23.5) assay was a modification of that of Barrett & Heath (1977). Sample (100 μl) and 400 μl 0·2 M-formate buffer (pH 3·0) were incubated with 250 μl 5% (w/v) haemoglobin for 30 min at 37°C. Protein was then precipitated by adding 1 ml trichloroacetic acid (100 g/l) and the mixture centrifuged at 14 000 ***g***. Controls were run similarly except that trichloroacetic acid was added immediately after haemoglobin. Enzymic activity was measured as the net absorbance at 280 nm of the supernatant in excess of that of the control. Lysozyme (EC 3.2.1.17) activity was measured as the ability to clear a suspension of *Micrococcus luteus*, using Barrett & Heath's procedure (1977). The assay was done at 23°C in 0·1 M-phosphate buffer (pH 6·3).

Assays for exoglycosidases were based on the ability of the enzyme to cleave the *p*-nitrophenyl derivatives of the relevant monosaccharide. For β-*N*-acetylglucosaminidase (EC 3.2.1.52) and β-*N*-acetylgalactosaminidase (EC 3.2.1.53), 100 μl sample and 700 μl 0·10 M-citrate buffer (pH 4·4) containing 0·01 M-NaCl and 0·2% (w/v) bovine serum albumin were incubated with 200 μl 5 mM-*p*-nitrophenyl derivatized monosaccharide for 30 min at 37°C. The reaction was terminated by adding 2 ml 0·5 M-carbonate buffer (pH 10·3). Free *p*-nitrophenol was determined by measuring absorbance at 410 nm. Other glycosidases were assayed by reacting 100 μl sample, 200 μl buffer and 200 μl 5 mM-substrate for 30 min at 37°C. Reactions were stopped with 1 ml 0·5 M-carbonate buffer (pH 10·3). Assay buffers were 0·2 M-citrate (pH 5·0) containing 5% (w/v) sodium taurocholate (α-galactosidase; EC 3.2.1.22), 0·2 M-citrate (pH 3·8) containing 0·1% (w/v) bovine serum albumin (β-galactosidase; EC 3.2.1.23), 0·2 M-citrate (pH 4·0) containing 0·6% (w/v) sodium taurocholate (β-glucosidase; EC 3.2.1.21), 0·2 M-acetate (pH 4·4) containing 0·1% (w/v) bovine serum albumin (β-glucuronidase; EC 3.2.1.31) and 0·2 M-citrate (pH 4·4) containing 10 mM-$ZnSO_4$ and 0·1% (w/v) bovine serum albumin (α-mannosidase; EC 3.2.1.24). Sodium taurocholate and $ZnSO_4$ were added because they stimulated enzymic activity.

Units. One unit of cathepsin D was defined as the ability to produce a net change of one A_{280} unit/min. Units of lysozyme were expressed in terms of activity of egg white lysozyme: 1 μg of the lysozyme standard produced a decrease in A_{600} of 0·015/min. For other enzymes, one unit was defined as the amount of enzyme causing production of 1 μmol product/min.

Protein determination. Protein in uterine flushings was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard. For determination of V_{max}, the procedure of Bradford (1976) was used, with lysozyme as a standard. Protein elution from columns was monitored by determining the A_{280} of the fractions.

Gel filtration. To determine the molecular weight of β-*N*-acetylglucosaminidase, 1 ml samples of uterine flushings were applied at 4°C to a Sephacryl S-200 column (77·0 × 1·5 cm), which had been calibrated with thyroglobulin, bovine serum albumin, ovalbumin, uteroferrin and ribonuclease (molecular weights of 660 000, 69 000, 43 000, 35 000 and 13 600 respectively). The column was equilibrated with 10 mM-Tris–HCl buffer (pH 8·2) containing 0·33 M-NaCl and 0·1 mM-phenylmethylsulphonyl fluoride (PMSF) or with 10 mM-phosphate buffer (pH 6·0) containing 0·2 M-NaCl and 0·1 mM-PMSF. Enzyme activity in fractions (2·9 ml) was monitored by enzyme assay.

Heat inactivation of glucosaminidase. Ion-exchange chromatography was used to separate acidic and basic isomers of β-*N*-acetylglucosaminidase. Uterine flushings (1 ml) were diluted with 9 ml 10 mM-citrate buffer (pH 6·6) and applied to a 3·0 × 0·75 cm column of CM–cellulose, pre-equilibrated with the same buffer. The acidic isomer did not bind to the column and eluted during washing of the column. The basic isomer was eluted with buffer containing 0·5 M-NaCl. Solutions of the isomers in 10 mM-phosphate buffer (pH 6·0) containing 0·5 M-NaCl were incubated at 50°C in a water bath. Aliquants were removed at 0, 15, 30, 60 and 120 min after immersion in the water bath and assayed for enzymic activity.

Chromatofocussing. The isolectric point of the acidic isomer of β-*N*-acetylglucosaminidase was determined by applying porcine uterine flushings (100 μl diluted with 900 μl 25 mM-histidine–HCl buffer, pH 6·2) to a 7·5 × 0·8 cm column of PBE-94 (Pharmacia) at 4°C, pre-equilibrated with 25 mM-histidine–HCl buffer, pH 6·2. After the sample entered the column, proteins were eluted with Polybuffer 74, pH 4·0 (Pharmacia), diluted 1:8 (v/v) with water. Application of this buffer to the column results in a pH gradient of 6·2 to 4·0. Fractions (2·8 ml) were collected and enzymic activity and pH determined. The isoelectric point of the basic isomer was determined in a similar manner except that starting buffer was 25 mM-ethanolamine–HCl (pH 9·4) and eluent was Polybuffer 96 (pH 7·0), diluted 1:10 (v/v) with water.

Binding to concanavalin A. To evaluate whether β-*N*-acetylglucosaminidase has mannose-containing oligosaccharides, 1 ml of porcine uterine flushings was diluted 1:10 (v/v) with 10 mM-phosphate buffer (pH 5·7) and applied at room temperature to a small (0·5 ml) column of concanavalin A–Sepharose 4B, pre-equilibrated with the same buffer. After washing the column with buffer containing 0·4 M-NaCl, enzyme was eluted by the sequential addition of 10% (w/v) α-methyl glucoside (in column buffer containing 0·4 M-NaCl) and 50 mM-α-methyl mannoside (in 0·1 M-acetic acid). Fractions were collected and those containing acetic acid were immediately diluted with 10 mM-phosphate buffer (pH 6·0).

CNAG–Sepharose chromatography. Procedures for coupling CNAG, a competitive inhibitor of β-*N*-acetylglucosaminidase, to Sepharose CL-4B and procedures for chromatography of uterine flushings were modified from those of Geiger & Arnon (1978). CNAG (20 mg) was mixed overnight at 4°C with 4 ml 0·1 M-carbonate buffer (pH 8·9) and 4 ml of a slurry of CNBr-activated Sepharose CL-4B. Sepharose beads were then collected by centrifugation and unreacted sites blocked by the addition of 2 ml 1 M-ethanolamine buffer (pH 9·0) for 2 h at room temperature. The coupling solution was reacted with an additional 14 ml CNBr-activated Sepharose. The two batches of CNAG–Sepharose were pooled, washed with column buffer (10 mM-phosphate, pH 6·0 with 25 mM-NaCl) and poured to form a 2·5 × 1·6 cm column.

A sample of endometrial culture medium (1 × 10^6 d.p.m.) was dialysed against column buffer and loaded onto the CNAG–Sepharose column. After washing with buffer, β-*N*-acetylglucosaminidase was eluted with column buffer (pH 7·0) containing 0·5 M-NaCl. Fractions (6 ml) were collected and assayed for enzymic activity and radioactivity.

Purification of glucosaminidase. An attempt was made to purify β-*N*-acetylglucosaminidase from uterine flushings of a pseudopregnant pig. Starting material was the protein fraction of uterine flushing that elutes at the void volume of Sephadex G-100 column. This material was

dialysed against 10 mM-Tris–HCl buffer (pH 8·2), run through a CM–cellulose column (20 ml, pH 8·2) to remove basic proteins and dialysed against 10 mM-phosphate buffer (pH 6·0). The preparation was then subjected to concanavalin A chromatography (using a 3 ml concanavalin A–Sepharose 4B column) and Sephacryl S-200 gel filtration using procedures similar to those earlier described. Fractions from the Sephacryl column having enzymic activity were pooled, dialysed against 10 mM-phosphate buffer (pH 6·0) containing 25 mM-NaCl and subjected to CNAG–Sepharose affinity chromatography as detailed earlier. After dialysis against 10 mM-phosphate buffer (pH 6·0), acidic and basic isomers were separated by CM–cellulose chromatography.

Immunoprecipitation. Samples (250 μl) of endometrial culture medium were incubated at 4°C overnight with 250 μl 50 mM-Tris–acetate buffer (pH 7·5) containing 0·3 M-NaCl, 1 mM-PMSF, 1 mM-EDTA, 0·1 mg bovine serum albumin/ml and 2% (v/v) Nonidet P-40 and with 10 μl rabbit anti-human hexosaminidase. Control incubations were with normal rabbit serum. Antigen–antibody complexes were adsorbed from the mixture by adding 100 μl of a 10% (v/v) slurry of Protein A–Sepharose CL-4B. After 6 h at room temperature on a tube turner, the mixture was centrifuged and the pellets washed five times with 50 mM-Tris–acetate buffer (pH 7·5) containing 0·3 M-NaCl, 0·5% (v/v) Nonidet P-40 and 0·1% (w/v) sodium dodecyl sulphate (SDS). Pellets were solubilized for electrophoresis by boiling for 2 min in 50 μl 5 mM-Tris–HCl buffer (pH 6·8) containing 10% (v/v) β-mercaptoethanol, 5% (w/v) SDS and 15% (v/v) glycerol. Antigen–antibody complexes were precipitated in other incubations by adding goat anti-rabbit IgG instead of Protein A and incubating overnight at 4°C.

Electrophoresis. One-dimensional electrophoresis using 10% (w/v) polyacrylamide gels was done according to the method of Laemmli (1970). Radioactivity was localized by fluorography using Kodak XAR-5 film after the gels were soaked in 1 M-sodium salicylate and dried.

Statistical analysis. Data presented in Table 1 and Text-fig. 1 were analysed by analysis of variance after log or log $(X + 1)$ transformation of the data to remove heterogeneity. When transformations could not remove heterogeneity, three orthogonal comparisons (oestradiol *versus* no oestradiol, progesterone *versus* no progesterone, and progesterone × oestradiol interaction) were made using Wilcoxon's two-sample test. Data in Table 2 were analysed by analysis of variance after log $(x + 1)$ transformation of the data. Means were compared using Duncan's new multiple range test. All statistical procedures used are detailed by Steel & Torrie (1960).

Results

Hormonal induction of enzymes in the pig uterus

Levels of various hydrolytic enzymes characteristically found in lysosomes were measured in uterine flushings of ovariectomized gilts treated with ovarian steroids (Table 1). Progesterone treatment increased ($P < 0{\cdot}05$ to $P < 0{\cdot}01$) uterine accumulation of all enzymes measured. Except for α-mannosidase, for which there was a decrease in enzymic activity, treatment with oestradiol-17β alone did not affect accumulation of lysosomal-like enzymes in the uterine lumen. When oestradiol was given in combination with progesterone, enzymic activities were reduced compared to activities in gilts treated with progesterone alone. This resulted in progesterone by oestradiol interactions for amounts of acid phosphatase ($P < 0{\cdot}05$).

Comparisons of enzymic activities in progesterone-treated animals and corn oil-treated animals indicated differential regulation of enzyme accumulation in the uterine lumen. For example, levels of acid phosphatase were 909-fold greater for progesterone-treated gilts than for corn oil-treated gilts. Progesterone caused greater than a 300-fold stimulation of β-*N*-acetylglucosaminidase and about a 179-fold stimulation of arylsulphatase. In contrast, there was only a 14-fold increase in β-glucuronidase activity and a 10-fold increase in β-glucosidase. Similarly, oestradiol-17β was a more

Table 1. Effect of progesterone and oestradiol-17β treatment on total accumulation of lysosomal enzymes in the uterine lumen of gilts*

	Treatment			
	Corn oil	Progesterone	Oestradiol-17β	Progesterone + oestradiol-17β
Protein (mg)	20 ± 9	408 ± 190	18 ± 1	99 ± 59
Acid phosphatase (U)	5 ± 2	4545 ± 3217	24 ± 11	357 ± 129
Arylsulphatase (U)	0·06 ± 0·03†	10·74 ± 2·50	0·02 ± 0·01†	4·33 ± 2·13
Cathepsin D (U)	0·20 ± 0·12†	30·9 ± 12·5	0·23 ± 0·15†	3·0 ± 1·2
Lysozyme (μg)	13 ± 13‡	374 ± 232	4 ± 4‡	177 ± 141†
β-*N*-Acetylglucosaminidase (U)	0·3 ± 0·1	91·1 ± 24·9	0·5 ± 0·1	55·2 ± 14·7
α-Galactosidase (U)	0·01 ± 0·01‡	4·10 ± 0·88	ND	1·85 ± 1·03
β-Galactosidase (U)	0·03 ± 0·01	130·14 ± 44·96	0·05 ± 0·03	7·43 ± 3·02
β-Glucosidase (U)	0·02 ± 0·01	0·20 ± 0·05	0·01 ± 0·004	0·05 ± 0·01
β-Glucuronidase (U)	0·02 ± 0·01	0·28 ± 0·03	0·01 ± 0·001	0·08 ± 0·06
α-Mannosidase (U)	0·65 ± 0·31	15·28 ± 5·55	0·14 ± 0·02	1·77 ± 1·26

* Values were derived by multiplying enzyme concentration in uterine flushings by volume of saline used to flush the uterus. Results are expressed as mean ± s.e.m. All effects of progesterone were significant ($P < 0·05$ to $P < 0·01$). Oestradiol treatment affected ($P < 0·05$) β-glucosidase and α-mannosidase activities. The progesterone × oestradiol interaction affected acid phosphatase ($P < 0·05$).

† Activity detectable for 2 of 3 animals. Non-detectable samples were given a value of zero for statistical purposes.

‡ Activity detectable for only 1 animal.

ND = non-detectable for all 3 animals.

potent antagonist of progesterone for some enzymes than for others. Activity of α-mannosidase in the uterine lumen of gilts treated with progesterone and oestradiol-17β was 12% of the activity for gilts treated with progesterone alone, while β-*N*-acetylglucosaminidase activity in animals treated with both steroids was 61% of the activity for progesterone-treated animals.

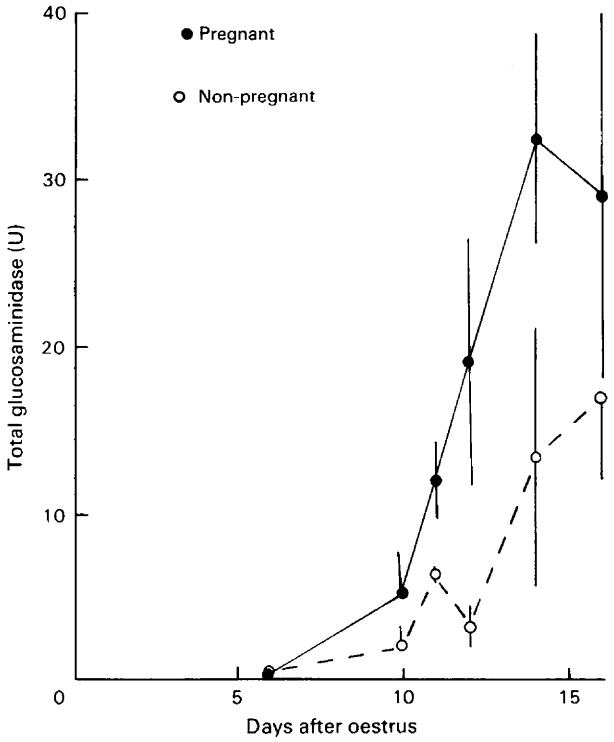
Enzymic activity at pH 7·0

Activities of enzymes at the pH optima were compared with activities at pH 7·0 to determine whether the enzymes would be potentially active in the uterine environment. Only one enzyme cathepsin D, was completely inactive at pH 7·0. At this same pH, several other enzymes (acid phosphatase, β-*N*-acetylglucosaminidase, α-galactosidase and α-mannosidase) retained less than 15% of their maximal activity. Activity of some other enzymes (arylsulphatase, β-galactosidase and β-glucosidase) at pH 7·0 was 20–27% of the activity of their pH optima. β-Glucuronidase retained 45% of its maximal activity and lysozyme was 57% as active at pH 7·0 as at pH 6·3, its pH optimum.

Activity of β-N-acetylglucosaminidase during the oestrous cycle, pregnancy and pseudopregnancy

Three enzymes, acid phosphatase, β-galactosidase and β-*N*-acetylglucosaminidase, were greatly elevated by progesterone treatment. Virtually all of the phosphatase activity in uterine flushings has been shown to be due to the presence of uteroferrin (A. T. Fazleabas, F. W. Bazer, P. J. Hansen, R. D. Geisert & R. M. Roberts, unpublished), an iron-containing protein with properties of a lysosomal phosphatase. We therefore investigated hormonal control and biochemical properties of β-*N*-acetylglucosaminidase in more detail.

Total β-*N*-acetylglucosaminidase in the uterine lumen of gilts during the oestrous cycle and early pregnancy is shown in Text-fig. 1. Enzymic activity increased ($P < 0·01$) with time after oestrus in pregnant and non-pregnant animals, reaching maximum levels at Days 14 and 16. Beginning at Day 10, β-*N*-acetylglucosaminidase was higher ($P < 0·02$) for pregnant than for non-pregnant gilts.



Text-fig. 1. Effect of stage of oestrous cycle and pregnancy on total uterine β-*N*-acetylglucosaminidase in gilts. Uteri were flushed on the days indicated and enzymic activity measured as described in 'Materials and Methods.' Total enzymic activity (enzyme concentration multiplied by volume of flush) was affected by days after oestrus ($P < 0{\cdot}01$) and pregnancy status ($P < 0{\cdot}02$). The day by pregnancy status interaction was not significant ($P > 0{\cdot}10$).

Activity of the enzyme was also measured in the uterine flushings of 4 pigs made 'pseudopregnant' by injecting oestradiol valerate daily from Days 11 to 15 after oestrus. This treatment results in prolonged maintenance of the corpus luteum and progesterone secretion. Uteri were flushed with 40–160 ml 0·9% (w/v) NaCl on Day 90 or 110 after oestrus and total luminal β-*N*-acetylglucosaminidase (enzyme concentration multiplied by volume of flush) averaged 146 ± 20 units (mean ± s.e.m.).

*Steroid induction of β-*N*-acetylglucosaminidase in mares and ewes*

Levels of β-*N*-acetylglucosaminidase were measured in uterine flushings or fluid of ovariectomized mares and ewes treated with steroids (Table 2). Treatment of mares with progesterone or oestradiol benzoate alone did not significantly alter enzyme accumulation in the uterine lumen. Administration of both steroids, however, stimulated ($P < 0{\cdot}05$) accumulation of the enzyme. Similar results were found for ewes, except that samples from progesterone-treated ewes were unavailable for analysis.

*Biochemical properties of β-*N*-acetylglucosaminidase*

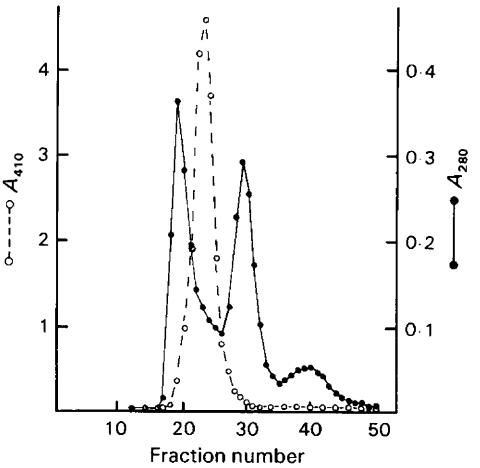
Molecular weight, pH optimum and Km. The molecular weight of the enzyme from pig uterine flushings was estimated by Sephacryl S-200 gel filtration (Text-fig. 2). Enzyme activity eluted as a

Table 2. Effect of progesterone and oestrogen on total uterine β-*N*-acetylglucosaminidase activity in ovariectomized mares and ewes*

Treatment	Mares		Ewes	
	No. of animals	Enzyme activity (U)	No. of animals	Enzyme activity (U)
Vehicle	4	0·04 ± 0·02[a]	2	0·50 ± 0·30[a]
Progesterone	3	0·44 ± 0·27[a,b]		
Oestrogen	5	0·04 ± 0·02[a]	3	1·26 ± 0·88[a]
Progesterone + oestrogen	4	2·18 ± 0·76[b]	3	16·1 ± 3·26[b]

* Results are expressed (mean ± s.e.m.) as total activity per uterus (mares) or per ligated uterine horn (ewes).

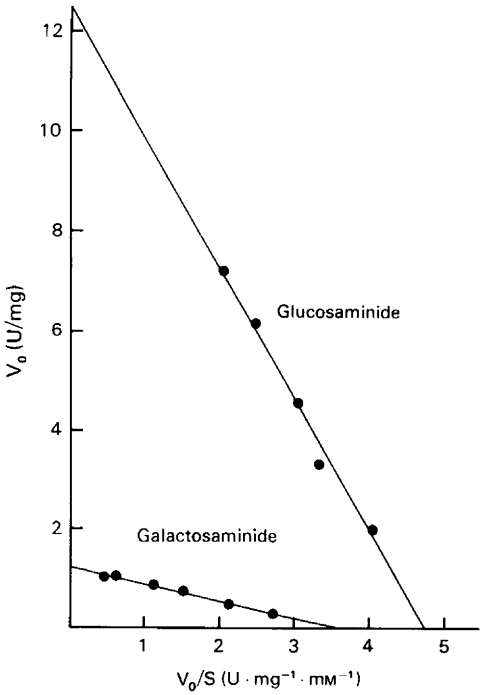
Means with different superscripts within a column differ ($P < 0·05$).



Text-fig. 2. Sephacryl S-200 chromatography of porcine uterine flushings. Uterine flushings (1 ml) were placed on a 77·4 × 1·5 cm column. Column buffer was 10 mM-Tris–HCl (pH 8·2) containing 0·33 M-NaCl and 0·1 mM-PMSF. Enzymic activity was measured in fractions (2·9 ml) as described in 'Materials and Methods'. An A_{410} of 1 equals 0·06 units of enzyme/ml. Protein was monitored by absorbance at 280 nm.

single peak with an apparent molecular weight of 82 000–89 000. The pH optimum of the enzyme was 4·4 when either *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide or *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide was the substrate. The *K*m was 2·62 mM for the former substrate and 0·32 mM for the latter substrate (Text-fig. 3). The V_{max} using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (12·5 units/mg crude protein) was greater than when *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (1·2 units/mg) was used.

Isomeric forms. Two isomeric forms of the enzyme exist. When a sample of pig uterine flushings was dialysed against 10 mM-citrate buffer (pH 6·6) and then placed on a column of CM–cellulose, 63% of the β-*N*-acetylglucosaminidase did not bind to the column, whereas the remaining 37% did and could be eluted with buffer containing 0·5 M-NaCl. Similarly, when uterine flushings were placed on a PBE 94 chromatofocussing column and eluted with a pH gradient of 6·4 to 4·0, two peaks of enzyme were detected, one at the void volume and one at pH 5·5. When eluted with a pH gradient of 8·3 to 6·8, 13% of the enzyme eluted at pH 8·0, while the rest remained bound to the



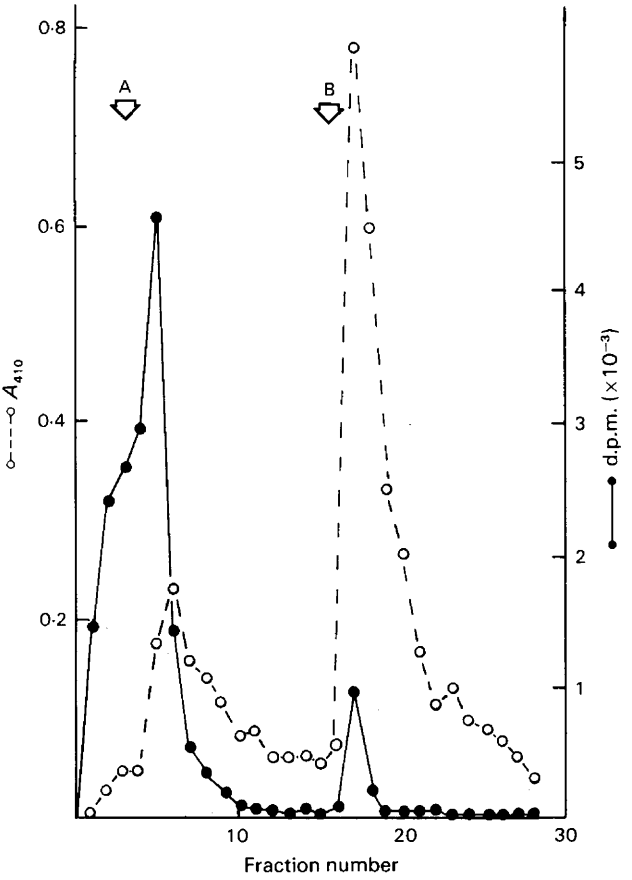
Text-fig. 3. Eadie–Hofstee plot of β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activity as a function of substrate concentration. Uterine flushings diluted in 0·10 M-citrate buffer (pH 4·4) were incubated with various concentrations of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (glucosaminide) or *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (galactosaminide) for 30 min at 37°C. Reaction volume was 1 ml and A_{410} was measured after adding 2 ml 0·5 M-carbonate buffer (pH 10·3). The *K*m (negative slope) was 2·62 mM for the former substrate and 0·32 mM for the latter substrate. The V_{max} (*y* intercept) using glucosaminide (12·5 U/mg crude protein) was greater than when galactosaminide was used (1·2 units/mg).

column. These results suggest the existence of isomers of the enzyme having isoelectric points of 5·5 and 8·0.

β-*N*-Acetylglucosaminidase isolated from other sources also exists as acidic and basic isoenzymes. According to several reports (Robinson & Stirling, 1968; Dance, Price, Robinson & Stirling, 1969; O'Brien, Okada, Chen & Fillerup, 1970), the basic enzyme is stable at 50°C, whereas the acidic isoenzyme is not. Large differences in heat stability were not noted for uterine β-*N*-acetylglucosaminidase, however. When placed at 50°C both isoenzymes lost 32–39% of their activity after 2 h.

Binding to concanavalin A and CNAG. The enzyme adsorbed to a column of concanavalin A–Sepharose 4B and 68% of the original activity was eluted by the sequential addition of 10% (w/v) α-methylglucoside (31% of the activity) and 50 mM-α-methylmannoside (37% of the activity). In another study, a competitive inhibitor, CNAG, was coupled to Sepharose 4B by cyanogen bromide activation. Porcine β-*N*-acetylglucosaminidase from uterine flushings or endometrial culture medium bound to this column at pH 6·0 and could be eluted with 10 mM-phosphate buffer (pH 7·0) containing 0·5 M-NaCl (Text-fig. 4).

Co-purification of glucosaminidase and galactosaminidase. We were unsuccessful in attempts to purify β-*N*-acetylglucosaminidase from pig uterine flushings using a 6-step purification procedure, largely due to the instability of the enzyme. The final yield of enzyme was only 7% of the original activity. The purification was 0·5-fold (acidic isomer) and 9·0-fold (basic isomer), suggesting that

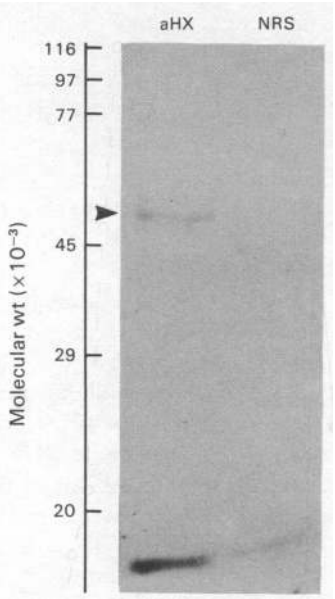


Text-fig. 4. Binding of β-*N*-acetylglucosaminidase to CNAG–Sepharose 4B. A sample of pig endometrial culture medium (1×10^6 d.p.m.) was applied to the column (2·5 × 1·6 cm). After washing with 10 mM-phosphate buffer (pH 6·0) containing 25 mM-NaCl (Buffer A), the enzyme was eluted with 10 mM-phosphate buffer (pH 7·0) containing 0·5 M-NaCl (Buffer B). Enzymic activity was measured in fractions (6 ml) as described in 'Materials and Methods'. An A_{410} of 1 equals 0·06 units of enzyme/ml.

loss of activity occurred during purification. The final preparations of enzyme contained several bands when subjected to electrophoresis. However, the ratio of β-*N*-acetylglucosaminidase to β-*N*-acetylgalactosaminidase (mean ± s.e.m. = 4·9 ± 0·15) remained constant throughout the purification, indicating that one molecule is probably responsible for both activities.

Secretion by porcine endometrium

To evaluate whether the endometrium synthesizes and secretes β-*N*-acetylglucosaminidase, endometrial tissue was cultured in the presence of [^{3}H]leucine and radioactive enzyme was isolated from the culture medium by immunoprecipitation with an antiserum against human β-hexosaminidase. A fluorograph of immunoprecipitated proteins separated by one-dimensional electrophoresis is shown in Text-fig. 5. A protein with a molecular weight of 48 000 was precipitated from the culture medium when antiserum was present, but not when normal rabbit serum was present. There was also a minor band (molecular weight 51 000) unique to the immunoprecipitate.



Text-fig. 5. Fluorograph of immunoprecipitates of [^{3}H]leucine-labelled endometrial proteins secreted into culture medium. Endometrium from a pseudopregnant gilt was cultured with L-[^{3}H]leucine. β-*N*-Acetylglucosaminidase was precipitated from the medium with rabbit anti-human hexosaminidase antiserum (aHX) or normal rabbit serum (NRS). Immunoprecipitates were subjected to one-dimensional, SDS electrophoresis using a 10% (w/v) polyacrylamide gel and radioactivity localized by fluorography. The arrow indicates a major band of radioactivity (mol. wt 48 000) that was present in precipitates of antiserum-treated medium but not in those in which normal rabbit serum was used. A minor band of mol. wt 51 000 was also precipitated with antiserum.

Discussion

The results reported here indicate that lysosomal enzymes accumulate in the uterine lumen through processes modifiable by steroid hormones. That the enzymes measured in uterine fluid are similar to enzymes found in lysosomes is indicated by several lines of evidence. All the enzymes examined have acidic pH optima, a feature of lysosomal enzymes (Barrett & Heath, 1977). Secondly, β-glucosidase is stimulated by sodium taurocholate while α-mannosidase activity is increased by the presence of zinc. These reagents have been shown to stimulate lysosomal β-glucosidase and α-mannosidase, respectively, but to inhibit cytoplasmic enzymes with the same hydrolytic actions (Marsh & Gourlay, 1971; Peters, Coyle & Glew, 1977).

Most importantly, β-*N*-acetylglucosaminidase in pig uterine flushings was found to have many properties similar to β-hexosaminidase found in lysosomes from several sources. These include similarities in molecular weight, pH optimum, *K*m, isoelectric point and binding to concanavalin A (Woolen, Heyworth & Walker, 1961; Robinson & Stirling, 1968; Dance *et al.*, 1969; O'Brien *et al.*, 1970; Geiger & Arnon, 1976, 1978; Srivastava, 1977). Based on the co-purification of β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities, one enzyme is probably responsible for both actions, as is true for the lysosomal enzyme (Woolen *et al.*, 1961; Robinson & Stirling, 1968; Geiger & Arnon, 1978). The molecular weight of the subunit of the enzyme secreted into culture medium by pig endometrium in this study is similar to the predominant subunit of β-hexosaminidase from human lysosomes found under reducing conditions (Geiger & Arnon 1976).

Taken together, these results indicate that the enzyme found in uterine flushings is similar to lysosomal β-hexosaminidase.

The appearance of lysosomal enzymes in the uterine lumen is a progesterone-induced event, modulated by oestrogens. In mares, oestrogen was synergistic with progesterone, while in gilts, oestrogen reduced the magnitude of the progesterone-induced accumulation of lysosomal enzymes. These differential effects probably reflect differences in dosage. Results of an experiment by Knight, Bazer, Wallace & Wilcox (1974), in which gilts were injected with various doses of oestradiol-17β in combination with progesterone, suggest that high doses of oestrogen inhibit progesterone induction of uterine protein secretion.

The finding that endometrial explants secrete immunoprecipitable, radiolabelled β-*N*-acetylglucosaminidase *in vitro* is evidence that at least some lysosomal enzymes in uterine fluid are endometrial secretory products. Uteroferrin is also released by the uterine endometrium (Basha *et al.*, 1979). Hormonal induction of secretion of lysosomal enzymes is not unique to the uterus as hormones also stimulate release of lysosomal hydrolases from bone cells (Vaes, 1969), the thyroid (Dingle, 1969) and the kidney (Brandt, Elliott & Swank, 1975).

Four enzymes in pig uterine flushings (acid phosphatase, β-galactosidase, β-*N*-acetylglucosaminidase and lysozyme) were present in greater amounts than the other enzymes. Glucosaminidase was also the most active enzyme found in cow and ewe uterine flushings (Roberts & Parker, 1974; Roberts, G.P. *et al.*, 1976a). Based on estimates of the specific activities of purified enzyme preparations when assayed by methods similar to those in the present study (Geiger & Arnon, 1978), the uterine lumen of progesterone-treated gilts contained an average of 61 mg acid phosphatase and 4 mg β-*N*-acetylglucosaminidase. The fact that stimulation of some enzymes by progesterone (and inhibition by oestrogen) was greater than for others suggests that steroids are causing differential gene expression (Palmiter, Mulvihill, McKnight & Senear, 1977), the endometrium is regionally differentiated with respect to protein secretion, or that the enzymes enter the uterus through different processes. Apart from secretion, lysosomal enzymes could enter the uterine lumen as a result of cell breakage or transudation from the blood.

The functions of lysosomal enzymes in the uterine lumen are unclear. Some of the enzymes are nearly inactive at the pH of the uterine lumen. Substantial hydrolysis of substrate could, however, occur over long periods. Roberts & Parker (1974) have postulated that exoglycosidases in uterine fluid modify glycoproteins in the plasma membrane of the trophoblast or endometrium to facilitate attachment of the conceptus to the uterine wall. Lysosomal enzymes could also be involved in breakdown of macromolecules to allow easier passage across the placenta. Lysozyme, which is perhaps lysosomal (Barrett & Heath, 1977), may have a bactericidal function (Cherry, Filler & Harvey, 1973). At least one-lysosomal-like enzyme, uteroferrin, has an iron-transport function *in utero* (Buhi *et al.*, 1982) which is distinct from its enzymic activity.

We thank A. M. Mejia, K. McDowell, R. J. Moffatt, A. T. Fazleabas and D. J. Sharp for donating samples of uterine flushings, S. Henry and C. Ketcham for technical help, G. A. Baumbach and P. T. K. Saunders for surgical assistance, Dr G. Sahagian and Dr E. Neufeld for donating antiserum to human β-hexosaminidase, Dr N. Sharon for providing CNAG, and J. Berceann for typing the manuscript. Research was supported by NIH Grant HD08560 and the Morris Animal Foundation. This is Journal Series No. 5635 of the Florida Agricultural Experiment Station.

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Received 18 June 1984